THE PHYSIOLOGY OF DIGESTION OF PLANKTON CRUSTACEA

I. Some Digestive Enzymes of Daphnia

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This paper constitutes the first report of a biochemical assay of the digestive enzymes of *Daphnia*.

The food of plankton Crustacea has been a contestable subject since Pütter (1909) postulated that most aquatic organisms derived much of their nutrition from dissolved organic matter. The experiments contrary to this theory culminated in the work of Krogh (1930), who found dissolved organic substances of no importance in the nutrition of aquatic animals and of Stuart, et al (1931), who raised bacteriologically sterile *Moina* and found them unable to subsist on dissolved nutritives —particulate food alone could furnish a supporting diet. "Staubfeine" detritus appeared to Naumann (1918) to play the most important rôle in the nutrition of cladocerans. He also (1921) determined a renewal coefficient of the intestinal contents (15–30 minutes) in several species of the same group which demonstrated the short period that food remains in their digestive tracts. Klugh (1927) found some Entomostraca able to utilize fine detritus but showed their chief food to consist of planktonic Chlorophyceae. Many workers point to the importance of bacteria as food. It is not unreasonable to suppose that they make up a part of the filterable food of plankton Crustacea, for Juday (1934) calculates them to be 1 per cent of the dry organic matter found in the centrifuge plankton of Trout Lake.

Birge and Juday (1922 and 1934), in a chemical study of particulate and dissolved organic matter in southeastern and 529 northeastern Wisconsin lakes, find considerable available food stuffs. In the latter group of lakes the organic matter in the centrifugable plankton consisted of 37 per cent crude protein, 4 per cent ether extract and 59 per cent carbohydrate. In the former report, analyses of algae show them to have a higher protein content than carbohydrate.

The experiments reported in this paper show that a digestive mechanism exists in *Daphnia* capable of utilizing protein, carbohydrate, and fat.

Acknowledgment is appreciatively given to Professor C. Juday for

suggesting the problem, to Professor H. C. Bradley, Department of Physiological Chemistry, under whose direction this work was carried out and who furnished laboratory facilities; also to Dr. H. D. Baernstein for instruction in the use of physico-chemical apparatus.

Materials and Methods

Daphnia magna was cultured in large battery jars and butter tubs, on a medium of sheep manure and acid phosphate. Frequent seining yielded sufficient *Daphnia* for limited analysis only. Pure cultures of *D. pulex* were netted from Lake Monona in the spring and fall of the year. Large quantities were obtained from this source.

Finely ground casein, on which the indicators neutral red and brom-cresol-phenol had been adsorbed, was fed to *D. magna*. The color change of the indicator was observed in progress through the alimentary tract and the pH approximated. The pH was found to vary from 6.8 in the anterior end of the tract to 7.2 at the caudal end. This result indicated a tryptic type of proteolytic digestion. Rankin (1929) found a range of 6.8–8.0 in *Simocephalus*.

In the preparation of the extract, *D. pulex* were strained from the water, dehydrated and partially defatted by treatment with acetone. Defatting was continued with petroleum ether. One gram of residue was powdered in an agate mortar and extracted for twenty-four hours with 100 cc. of 50 per cent glycerol. The filtered extract contained proteinases, carbohydrate and fat-digesting enzymes. Fresh hog pancreas with a strip of duodenum were treated in the same manner as the *Daphnia*. This extract was compared with *Daphnia* extract in some of the experiments. For micro-analysis 50 intestines were dissected from *D. magna* and extracted with 50 per cent glycerol.

Examination of the Extract

The Proteinases

In order to micro-analytically detect the presence of a proteinase in *D. magna*, the Gates (1927) photographic plate method was modified to show qualitatively the proteolytic activity of the glycerol extract of *D. magna* intestines. Drops of this extract definitely digested the gelatin film of a photographic plate showing the presence of a proteinase in the digestive tract of *Daphnia*.

For quantitative analysis of the *Daphnia* proteinase, the viscosity method of Northrop (1922) was modified for use. With the aid of the Ostwald viscosimeter, it was possible to follow the hydrolysis of gelatin by a proteinase. The activity of the enzyme is measured in terms of

decreasing viscosity ($\triangle$V) of the substrate. A special gelatin from Swift and Co. was used for the first experiments. Solutions of 3 per cent concentration were made up in a phosphate buffer at pH 7.4, preserved in thymol and kept in test tubes (5 cc./tube) at 3° C. Test tubes of gelatin were placed in a water bath of 34° C.; the temperature was kept constant by a heating unit, agitator, and toluene regulator. When the gelatin reached bath temperature, it was transferred to Ostwald viscosimeters. To these were added 0.5 cc. of the 1 per cent glycerol extract. Viscosity readings were taken as often as possible for the first 15 minutes. After that, readings were made at 10-minute

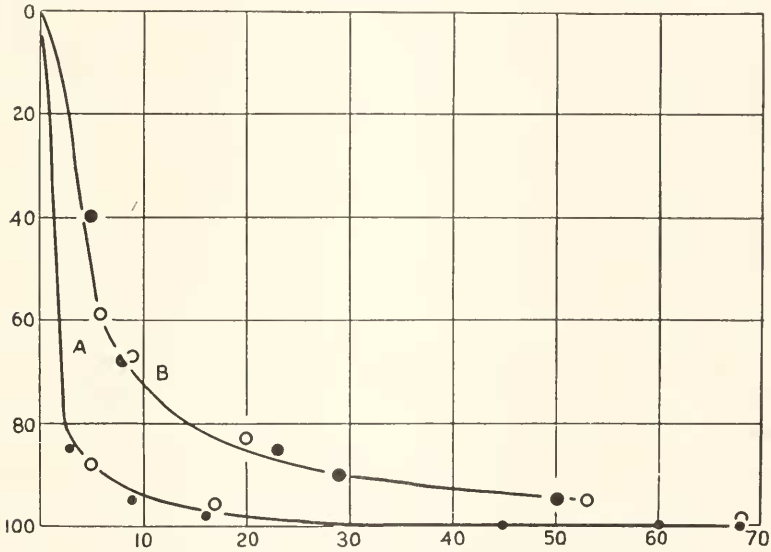

FIG. 1. Hydrolysis of gelatin at pH 7.4 by proteinases of hog pancreas (*A*), and of *Daphnia* (*B*). The ordinate represents decreasing viscosity ($\triangle$V); the abscissa is the time in minutes.

intervals for one hour. To the control tubes was added 0.5 cc. of 50 per cent glycerol. The gelatin-digesting ability of 1 per cent glycerol extracts of both *D. pulex* and hog pancreas were determined and compared. Fig. 1 shows the hydrolysis of gelatin by the proteinases in the extracts of both *Daphnia* and hog pancreas. The curves represent the mean of two runs against a control and were duplicated twice. They are typical hydrolysis curves, falling suddenly at the onset of cleavage. The curves then level off, but *A* gradually approaches *B*. The 1 per cent extract of *Daphnia* decreased 84$\triangle$V in 20 minutes while a 1 per cent extract of hog pancreas fell to 100$\triangle$V in the same time.

Effect of pH on Enzyme Activity

The character of an enzyme is determined by the pH of optimum activity. Mammalian peptic enzymes are most active at pH 1.0, tryptic enzymes pH 7–8 and katheptic enzymes pH 4–5. The subsequent procedure was followed in determining the effect of pH upon proteinase activity of the *Daphnia* extracts. The 1 per cent extract used in the above experiment was diluted to contain 1.5 *Daphnia* proteinase units. A unit was defined as the amount of enzyme necessary to cause a decrease of 20△V of 1.5 per cent Sargent's gelatin in 20 minutes; pH 7.4, temperature 34° C.

Test tubes of 1.5 per cent Sargent's gelatin were buffered in a series at hydrogen-ion concentrations of 1–10 and preserved with thymol. The pH was determined with the aid of the quinhydrone electrode. To

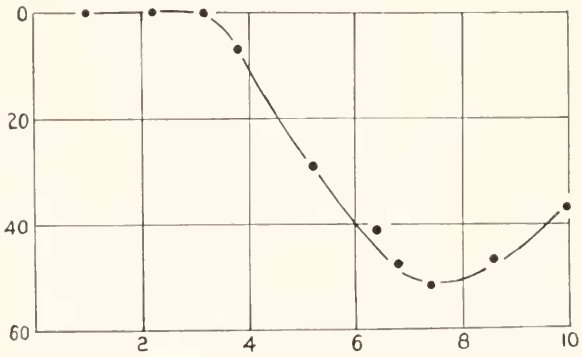

Fig. 2. Activity of *Daphnia* proteinases—effect of pH on gelatin hydrolysis. Decreasing viscosity (△V) is the ordinate; the abscissa represents pH.

5 cc. of gelatin at a desired pH, 0.5 cc. of enzyme solution containing 1.5 units was added. Viscosity readings were made and a graph constructed for proteinase activity. The readings at the end of 50 minutes, for each of the pH 1–10 runs, were taken and plotted against pH. The activity of the proteinase at any pH can be read from Fig. 2. The optimum activity was reached at pH 7.4. Its activity decreased on the alkaline side of pH 7.4. On the acid side, the enzyme was found to be inactive at pH 3.2. The graph was constructed from the mean of two runs at each pH. A control was used in all cases and the experiment duplicated three times.

Amylase

The amount of maltose produced by the action of 1 per cent extract on a 3 per cent corn starch solution was iodometrically titrated by the

Baker and Hulton (1920) method for the estimation of sugars and used as an indication of amylase activity. A 10-cc. sample starch solution, pH 7.4, was diluted to 50 cc. and used as the substrate. To this was added 1 cc. of extract and thymol for preservative, and it was then kept at 37° C. Every 15 minutes 5-cc. samples were withdrawn and titrated. The increase in the amount of thiosulphate used in the titration was equivalent to the same amount of maltose liberated in the digest. Duplicate samples were used. The curves in Fig. 3 represent the mean of two runs. The control showed no hydrolysis during the run.

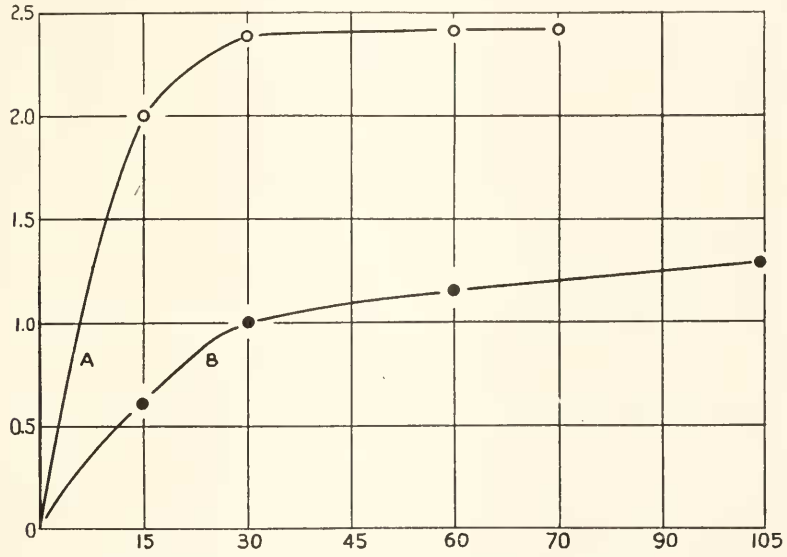

FIG. 3. Hydrolysis of starch at pH 7.4 by amylase of hog pancreas (*A*), and of *Daphnia* (*B*). The ordinate represents the number of cc. of N/20 maltose; the abscissa is the time in minutes.

The concentration of amylase is much greater in the glycerol extract of hog pancreas than in the *Daphnia*. After 30 minutes the hydrolysis of the starch by 1 per cent hog pancreas extract was equivalent to 2.4 cc. of N/20 maltose, while in the same time 1.0 cc. was the total amount produced by the *Daphnia* extract.

LIPASE

For the determination of lipase activity, 50 cc. of 4 per cent tributerine were emulsified with sodium glycocholate. To this were added 2 cc. of 1 per cent extract. The digest was placed at 37° C. and 10-cc. samples were withdrawn hourly and titrated with N/20 NaOH. The

increase in acidity due to the liberation of fatty acids by the hydrolytic action of the lipase in the *Daphnia* extract and hog pancreas extract was recorded in terms of N/20 NaOH. The results are shown in Fig. 4. The experiments were duplicated and run with controls. The presence of a lipase in the *Daphnia* extract is clearly demonstrated. The mean acidity at the end of four hours was equivalent to 0.9 cc. of N/20 NaOH in the case of 1 per cent glycerol extract of *Daphnia* and 2.6 for hog pancreas.

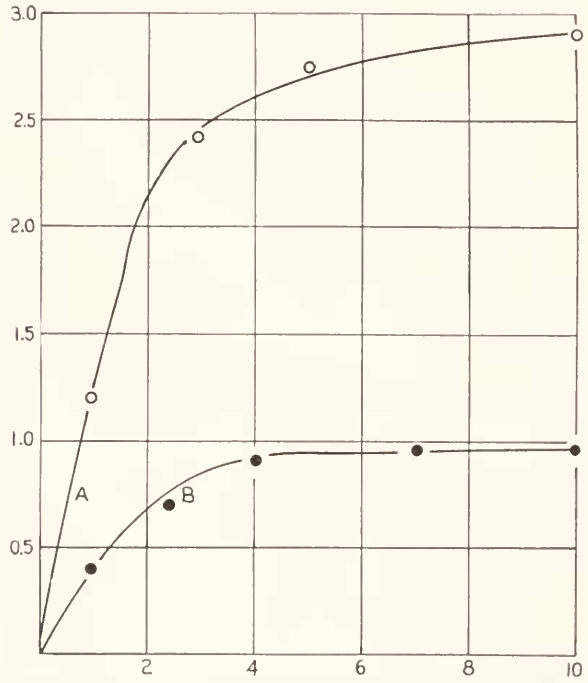

Fig. 4. Hydrolysis of tributerine by lipase of hog pancreas (*A*), and of *Daphnia* (*B*). The ordinate represents the number of cc. of N/20 NaOH; the abscissa is the time in hours.

Discussion

The experiments show the presence of a proteinase, amylase, and lipase which partially complete the enzyme mechanism for handling proteins, carbohydrates, and fats available to *Daphnia* in its natural habitat and under natural feeding conditions. These experiments show that the activities of the proteinase and amylase are such that considerable digestion is possible in the 15–30 minutes that food is said to remain in the alimentary tract. From Fig. 1 it was computed that 88 per

cent of the total digestion of gelatin done by a 1 per cent extract of *Daphnia* was completed within 20 minutes. Fig. 3 shows that of the total amount of maltose formed by amylase in 75 minutes, 80 per cent was produced in the first 30 minutes.

Figure 1 illustrates a comparison of the proteolytic activity of a 1 per cent glycerol extract of defatted *D. pulex* and a 1 per cent glycerol extract of defatted hog pancreas. It is apparent that the hog pancreas extract contains more enzyme than the *Daphnia* extract, due primarily to the fact that the digestive enzymes of the hog are most concentrated in the pancreas. On the other hand, however, if the amount of proteinase present per body weight of *Daphnia* be compared with the amount per body weight of hog, the picture would look very different. Estimating a hog to weigh 200 lbs., the hog pancreas 0.5 lbs., and the average *Daphnia* 0.0043 oz., rough calculations approximate the amount of proteinase to be from 300 to 400 times greater per body weight of *Daphnia* than per body weight of hog.

The proteinase obtained by gross extraction of the entire organism is found to have an optimum digestion at pH 7.4. This extract may obviously contain proteases other than the digestive enzymes, and any interpretation of the results may be made with this in view. Isolation of a proteinase from the digestive tract of *Daphnia* was not attempted. On the other hand, the extracts of 50 isolated digestive tracts of *D. magna* showed definite proteolytic activity at pH 7, which corresponds with the pH observed in the digestive tract and with the optimum pH of the enzyme mixture obtained from the entire animal. It will be recalled that mammalian tissue proteases react best in slightly acid media-about pH 4 +, and not at all at 7. The author believes, therefore, that he is warranted in assuming that the proteinase obtained by extracting the entire *Daphnia,* acting best at pH 7.4, is the digestive enzyme of the alimentary tract.

The character of the proteinase simulates that of mammalian trypsin and the initial cleavages on gelatin were undoubtedly produced by this tryptic-like proteinase, since Waldschmidt-Leitz (1929) holds that erepsin does not hydrolyze gelatin. It is very probable, however, that erepsin was present and played some part in the splitting of the poly- and di-peptides after the initial cleavages by the proteinase had been made. In addition, the fact that the extract was practically inactive at pH 3.8 and completely inactive at 3.2 rules out the possibility of the presence of a peptic type of digestion.

Summary

1. A digestive enzyme system exists in *Daphnia* that enables it to digest considerable protein and carbohydrate within 30 minutes.

2. Microchemical analysis of glycerol extracts of *D. magna* intestines demonstrated the presence of a proteinase similar to trypsin. Quantitative estimations were made of proteinase activity of whole *D. pulex* extracts.

3. No pepsin was found in the extracts.

4. Amylase and lipase were chemically demonstrated in extracts of *Daphnia*.

5. The pH of the alimentary tract of *D. magna* was found to range from 6.8–7.2.

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